Solid-Phase Synthesis of 2′-Deoxy-2′-fluoro-β-D-Oligoarabinonucleotides (2′F-ANA) and Their Phosphorothioate Derivatives

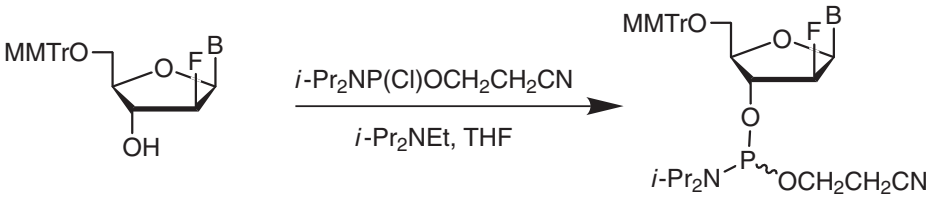
This unit describes the chemical synthesis of 2′-deoxy-2′-fluoro-β-D-oligoarabinonucleotides (2′F-ANA), both with phosphodiester and phosphorothioate linkages. The protocols described herein include araF phosphoramidite preparation (see Basic Protocol 1), assembly on DNA synthesizers (see Basic Protocol 2), and final deprotection and purification of oligonucleotides (see Basic Protocol 3).

The preparation of araF phosphoramidite building blocks is carried out by introducing a 2-cyanoethyl-*N*,*N*-diisopropylaminophosphinyl group at the 3′-*O*-position of conveniently protected araF nucleosides. The preparation of araF-protected nucleosides is described in *UNIT 1.7*.

Assembly of 2′F-ANA sequences can be carried out under similar conditions as for DNA sequences, but longer coupling times for ara F phosphoramidite monomers are required. For phosphorothioate analogs, longer sulfurization times are also required, as compared to *S*-DNA synthesis.

Assembly of the oligonucleotides is carried out by the stepwise addition of phosphoramidite building blocks to nucleoside or nucleotide hydroxyl termini preimmobilized on a solid support until the desired sequence is obtained (*APPENDIX 3C*). Each addition of new building block requires five steps: detritylation, coupling, capping, oxidation or sulfurization, and capping. The last capping reaction also serves to dry the column prior to the next coupling cycle. Cleavage of the sequence from the solid support and the removal of the nucleobase- and phosphodiester-protecting groups is carried out using an aqueous ammonia/ethanol mixture. Crude oligonucleotides obtained in this way can be purified by ion-exchange high-performance liquid chromatography (HPLC; *UNIT 10.5*) or denaturing polyacrylamide gel electrophoresis (PAGE; *UNIT 10.4*).

NOTE: All glassware should be oven dried.



1a B = N^6-benzoyladenin-9-yl
b B = N^2-isobutyrylguanin-9-yl
c B = N^4-benzoylcytosin-1-yl
d B = thymin-1-yl

2a (75%)
b (70%)
c (80%)
d (95%)

Figure 4.15.1 Synthesis of the four araF-protected nucleoside phosphoramidites **(S.2a-d)**. MMTr, 4-monomethoxytrityl; *i*-Pr_2NEt, *N*-ethyl-*N*,*N*-diisopropylamine; *i*-Pr_2-NP(Cl)OCH_2CH_2CN, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite.

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Current Protocols in Nucleic Acid Chemistry (2002) 4.15.1-4.15.22

BASIC PROTOCOL 1

PREPARATION OF araF PHOSPHORAMIDITES

Conversion of protected araF nucleosides into the corresponding phosphoramidite building blocks is shown in Figure 4.15.1 (Wilds and Damha, 2000). In order to obtain phosphoramidites in sufficiently high yields, the starting materials and reaction solvents should be as dry as possible and all glassware should be oven dried. Likewise, to simplify purification, the reaction should be completed using a minimal amount of phosphitylation reagent.

Materials

Protected araF nucleosides (*UNIT 1.7*):
- N^6-Benzoyl-9-[2-deoxy-2-fluoro-5-*O*-(4-methoxytrityl)-β-D-arabinofuranosyl] adenine (**S.1a**)
- N^2-Isobutyryl-9-[2-deoxy-2-fluoro-5-*O*-(4-methoxytrityl)-β-D-arabinofuranosyl] guanine (**S.1b**)
- N^4-Benzoyl-1-[2-deoxy-2-fluoro-5-*O*-(4-methoxytrityl)-β-D-arabinofuranosyl] cytosine (**S.1c**)
- 1-[2-Deoxy-2-fluoro-5-*O*- (4-methoxytrityl)-β-D-arabinofuranosyl]thymine (**S.1d**)

THF, anhydrous (see recipe)
N-Ethyl-*N,N*-diisopropylamine (DIPEA; Aldrich), double distilled
2-Cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (Chem Genes)
Dichloromethane
1:9 and 1:19 (v/v) methanol/dichloromethane
Saturated sodium bicarbonate solution
Magnesium sulfate, anhydrous
Solvent system:
- 1:99 (v/v) triethylamine/chloroform (for araF-T)
- 1:9:10 (v/v) triethylamine/dichloromethane/hexanes (for araF-A and araF-C)
- 1:99 (v/v) triethylamine/dichloromethane (for araF-G)

Silica gel (230 to 400 mesh)
Diethyl ether

50-mL round-bottom flask equipped with stir bar and rubber septum
Syringe
TLC Merck silica plates (Kieselgel 60 F-254)
254-nm UV lamp
500-mL separatory funnel
Vacuum evaporator (e.g., Savant Speedvac)
3-cm-diameter chromatography column

Additional reagents and equipment for TLC (*APPENDIX 3D*) and column chromatography (*APPENDIX 3E*)

Prepare araF phosphoramidite monomers

1. In a 50-mL round bottom flask equipped with a stir bar and rubber septum, dissolve 1 mmol of each protected araF nucleoside (**S.1a-d**) in 3 to 5 mL of anhydrous THF.

2. While stirring, add 0.63 mL (3.6 mmol) double distilled *N*-ethyl-*N,N*-diisopropylamine (DIPEA) followed by the slow addition of 0.24 mL (1.1 mmol) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite.

   A white precipitate of diisopropylethylammonium hydrochloride salts forms after 10 min and is indicative of sufficiently anhydrous conditions for a successful reaction.

3. Stir the reaction mixture for an additional 2 hr or until complete consumption of starting material is observed. For TLC analysis (*APPENDIX 3D*), remove 10 to 20 µL reaction mixture by syringe and dilute with 100 µL dichloromethane in a small tube. Analyze on a TLC Merck silica plate using 1:19 (v/v) methanol/dichloromethane for araF-T, -C, and -A, and 1:9 (v/v) methanol/dichloromethane for araF-G. Co-spot the starting material for comparison. Visualize by exposure with a 254-nm UV lamp.

   TLC solvents in this step do not include TEA. However, TLC solvents listed under step 11, can be substituted for those listed here.

   The phosphoramidites migrate faster than the starting material. In this reaction, two stereoisomeric products are formed, but they are not separable in the methanol/dichloromethane system.

4. If, as demonstrated by TLC, the reaction is not complete (i.e., more than ~5% of starting material remaining), add an additional portion (~0.2 eq.) of 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, stir for 2 hr more, and repeat TLC analysis (step 3). If the reaction looks complete by TLC analysis, start workup of the phosphoramidite.

   It is important to minimize the amount of phosphitylating reagent used, otherwise the excess is hydrolyzed during the workup to form H-phosphonate impurities (^{31}P NMR: singlet, ~14 ppm), which can cause difficulties during subsequent purification of the amidites.

5. Dilute the reaction mixture with 150 mL dichloromethane, transfer it to a 500-mL separatory funnel, and wash with 150 mL saturated sodium bicarbonate solution.

6. Collect the organic layer, dry it by adding solid anhydrous magnesium sulfate until it no longer clumps, and filter and concentrate under reduced pressure to obtain a crude product.

   Chromatographic purification of the crude amidite is recommended, but if the reaction mixture looks pure by TLC and evaporates easily to give a stable foam, this step may be omitted.

   In case of a second day purification, it is strongly recommended to evaporate the crude compound to a foam, dry it under high vacuum, and keep the product in a vacuum desiccator. If the product persists as an oil after these steps are taken, co-evaporate it with diethyl ether. A foam can usually be obtained after co-evaporation.

Purify phosphoramidite monomers by flash column chromatography

7. Redissolve the crude material in a minimal amount of one of the following solvent systems:

   1:99 (v/v) triethylamine/chloroform for araF-T
   1:9:10 (v/v) triethylamine/dichloromethane/hexanes for araF-A and araF-C
   1:99 (v/v) triethylamine/dichloromethane for araF-G.

8. Apply sample to a 3-cm-diameter chromatography column containing 50 g 230- to 400-mesh silica gel, preconditioned with the appropriate chromatography solvent (step 7).

   Column chromatography is performed according to the method of Still et al. (1978) using a small amount of air pressure at a rate of ~1 inch of solvent per minute (also see APPENDIX 3E).

9. Apply the sample to the column and begin eluting with the preconditioning solvent.

   a. *For araF-C and araF-A:* Elute in 1:9:10 (v/v) triethylamine/dichloromethane/hexanes.

   b. *For araF-T:* Follow a stepwise gradient of triethylamine/chloroform until a 1:31 ratio is reached. For 1 mmol crude sample, collect 100 mL eluate at 1% TEA, 100 mL at 2% TEA, and so on.

   c. *For araF-G:* Follow a step gradient of methanol/triethylamine/dichloromethane from 0:1:99 to 5:1:94 (v/v). For 1 mmol crude sample, collect 100 mL at 0% methanol, 100 mL at 1% methanol, and so on.

   Always add at least 0.5% of triethylamine to the chromatographic solvent mixtures used for elution and preconditioning of the column in order to avoid hydrolysis of the amidites to the H-phosphonates during purification. Step gradients indicated are only intended as guidelines and should be adjusted according to various factors including amount of crude product applied and flow rate of mobile phase.

10. Monitor fractions by TLC (*APPENDIX 3D*; step 3) and pool those containing pure phosphoramidite.

11. Evaporate the phosphoramidite on a vacuum evaporator and dry under high vacuum to obtain a stable foam. If a foam does not form, try co-evaporation of the pure product with diethyl ether.

    Typically, a colorless or pale yellow foamy product forms easily. This can be stored in a vacuum desiccator over phosphorus pentoxide or at −20°C in a freezer for several months without decomposition.

    *For araF-A (**S.1a**), the typical yield after chromatographic purification is ~70% to 75%. TLC using 5:45:50 (v/v/v) triethylamine/hexanes/dichloromethane results in two spots corresponding to two isomers at R_f 0.22 and 0.33. ^{31}P NMR (202.3 MHz, acetone-d_6): 150.9 and 151.1, ^{19}F NMR (470.27 MHz, acetone-d_6, without external reference): yields −197.3 and −197.7 (ddd, $J_{1'\text{-}F}$ = 19 Hz, $J'_{2'\text{-}F}$ = 52 Hz, $J_{3'\text{-}F}$ = 19 Hz), and FAB-MS using an NBA matrix yields 846.34 [M^+].*

    *For araF-G (**S.1b**), the typical yield after chromatographic purification is ~70% to 75%. TLC using 20:1 (v/v) chloroform/methanol yields a spot of R_f 0.54; ^{31}P NMR (200.06 MHz, acetone-d_6, 85% ortho-phosphoric acid as external reference): 151.8 and 151.0; ^{19}F-NMR (282.32 MHz, acetone-d_6, 99% trifluoroacetic acid as external reference): −119.7 and −119.4; and FAB-MS using an NBA matrix yields 822 [M^+].*

    *For araF-C (**S.1c**), the typical yield after chromatographic purification is ~80% to 85%. TLC using 5:45:50 (v/v/v) triethylamine/hexanes/dichloromethane yields two spots, corresponding to two isomers, at R_f 0.26 and 0.34; ^{31}P NMR (500 MHz, acetone-d_6): 151.3 and 150.8; ^{19}F NMR (470.27 MHz, acetone-d_6, without external reference): −199.0 and −199.2 (ddd, $J_{1'\text{-}F}$ = 18 Hz, $J'_{2'\text{-}F}$ = 52 Hz, $J_{3'\text{-}F}$ = 18 Hz); and FAB-MS using an NBA matrix yields 828 [M^+].*

    *For araF-T (**S.1d**), typical yield after chromatographic purification is ~90% to 95%. TLC using 10:9:1 (v/v/v) chloroform/ethyl acetate/ethanol yields two spots, corresponding to two isomers, at R_f 0.76 and 0.83; ^{31}P NMR (500 MHz, acetone-d_6): 151.3 and 150.8; ^{19}F NMR (470.27 MHz, acetone-d_6, without external reference): −199.0, −199.2 (ddd, $J_{1'\text{-}F}$ = 18 Hz, $J'_{2'\text{-}F}$ = 52 Hz, $J_{3'\text{-}F}$ = 18 Hz); and FAB-MS using an NBA matrix yields 822 [M^+].*

SOLID-PHASE ASSEMBLY OF PROTECTED araF PHOSPHORAMIDITES

This protocol describes the setup and step-by-step synthesis of araF oligonucleotides. The methodology has been optimized for use with the Expedite 8909 DNA synthesizer equipped with a workstation (PerSeptive Biosystems), but can easily be adapted to other automated DNA synthesizers. All syntheses have been performed on 1-μmol scales, both for phosphodiester- and phosphorothioate-containing araF oligonucleotides. The coupling time for araF nucleosides requires a 15-min "wait" step, as compared to 1.5 min for deoxynucleosides on the Expedite instrument. Likewise, the detritylation time has been extended to 150 sec to allow for effective removal of the monomethoxytrityl (MMTr) protection as opposed to the more labile dimethoxytrityl (DMTr) groups that are commonly used with standard DNA monomers. For thio-araF-oligonucleotide synthesis, the overall success of oligomer synthesis was evaluated with different sulfur-transfer reagents, including 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent; Iyer et al., 1990), 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH; Xu et al., 1996), and 3-amino-1,2,4-dithiazoline-5-thione (ADTT; Tang et al., 2000). In all cases, the concentration of sulfurization reagent was increased ~5 fold as compared to customized S-DNA synthesis, and the time required for the oxidation was prolonged to 10 min as opposed to 1 to 2 min for S-DNA synthesis. Current procedures can easily be adapted to the synthesis of molecules with mixed nucleotide composition (e.g., DNA/araF chimeras of variable gap sequences).

Materials

AraF phosphoramidites (see Basic Protocol 1)
Argon gas
Acetonitrile, anhydrous (see recipe)
Liquid Reagent kit for the Expedite 8909 instrument (PerSeptive Biosystems):
- Acetonitrile wash and amidite diluent: anhydrous acetonitrile
- Activator solution: dissolve 1.8 g sublimed tetrazole (0.5 M) in 50 mL acetonitrile; store up to 2 wks at room temperature.
- Cap A (see recipe)
- Cap B (see recipe)
- Oxidizer solution (see recipe)
- Deblock solution: 15 g trichloroacetic acid in 500 mL dichloromethane; store up to 1 yr at room temperature

Sulfurization reagent (see recipe)
Amidite column (see recipe)

Synthesizer vials with caps
Vacuum desiccator containing phosphorus pentoxide
Syringe
Automated DNA synthesizer (e.g., Expedite 8909, Perseptive Biosystems) with trityl monitor

Additional reagents and equipment for oligonucleotide synthesis (*APPENDIX 3C*)

Synthesize phosphodiester and phosphorothioate araF oligonucleotides

1. Calculate the amount of araF phosphoramidites required for the synthesis of a desired sequence.

   The typical concentration of amidites used with the Expedite synthesizer corresponds to 50 mg/mL. Note that the volume of amidite solution delivered to the column during each coupling step is ~240 μL. However, an excess of 50 mg amidite (i.e., 1 mL solution) should be included when preparing each amidite solution in order to purge the lines of the synthesizer.

Table 4.15.1 Automated 1 µmol Synthesis Cycle for the Synthesis of Phosphodiester araF Oligonucleotides on the Expedite 8909 Instrument[a]

Function[b]	Amount[c]	Time (sec)	Description
Detritylation of the support bound nucleoside:			
Trityl monitor on/off	—	1	Trityl analysis monitor is turned on for data collection
Deblock	10	0	Detritylation solution is rapidly delivered to column
Deblock	70	150	Slow, prolonged delivery of detritylation solution to column
Diverted wash A	40	0	Lines are flushed with anhydrous acetonitrile (wash A)
Trityl mon. on/off	—	1	Trityl quantitation is stopped
Diverted wash A	40	0	Lines are flushed with anhydrous acetonitrile
Coupling of phosphoramidite:			
Wash	5	0	Lines are flushed with acetonitrile
Activator	5	0	Lines are flushed with tetrazole solution
A + activator	6	0	Rapid addition of the phosphoramidite (A) and tetrazole solutions to the column
A + activator	9	500	Coupling of the free activated phosphoramidite to the support-bound terminal nucleoside
Wash	8	400	Slow delivery of acetonitrile to the column to purge remaining phosphoramidite solution
Wash	7	0	Lines and column are washed with acetonitrile
Capping of the column:			
Wash A	20	0	System is flushed with anhydrous acetonitrile
Cap A and B	7	0	Equal volumes of cap A and cap B solutions are rapidly delivered to column
Cap A and B	6	15	Slower delivery of cap A and cap B to column (to maximize capping efficiency)
Wash A	6	15	Slow delivery of acetonitrile to column to purge remaining cap A and B solutions
Wash A	14	0	Lines and column are flushed with anhydrous acetonitrile
Oxidation of phosphoramidite:			
Oxidizer	20	0	Oxidant is delivered to the column to oxidize the newly formed phosphite triester linkages
Wash A	15	0	Lines and column are flushed with anhydrous acetonitrile
Capping of the column:			
Cap A and B	7	0	Equal volumes of cap A and B solutions are rapidly delivered to column
Wash A	30	0	Lines and column are flushed with anhydrous acetonitrile

[a]Standard DNA coupling cycles supplied by the manufacturer have been optimized for 2′-araF oligonucleotide assembly.
[b]All entries are as described in a typical cycle sequence for the Expedite instrument; see manufacturer's directions for further information.
[c]Represents the number of pulses required for the corresponding step; each pulse has approximately 16 µL volume.

Table 4.15.2 Automated 1-μmol Synthesis Cycle for the Synthesis of Phosphorothioate araF Oligonucleotides on Expedite 8909 Instrument[a]

Function	Amount[b]	Time (sec)	Description
Detritylation of the support-bound nucleoside:			
Trityl monitor on/off	1	1	Trityl analysis monitor is turned on for data collection
Deblock	10	0	Detritylation solution is rapidly delivered to column
Deblock	70	150	Slow, prolonged delivery of detritylation solution to column
Diverted wash A	40	0	Lines are flushed with anhydrous acetonitrile (wash A)
Trityl monitor on/off	0	1	Trityl quantitation is stopped
Diverted wash A	40	0	Lines are flushed with anhydrous acetonitrile
Coupling of phosphoramidite:			
Wash	5	0	Lines are flushed with acetonitrile
Activator	5	0	Lines are flushed with tetrazole solution
A + activator	6	0	Rapid addition of the phosphoramidite (A) and tetrazole solutions to the column
A + activator	9	500	Coupling of the free activated phosphoramidite to the support-bound terminal nucleoside
Wash	8	400	Slow delivery of acetonitrile to the line to purge remaining phosphoramidite solution
Wash	7	0	Lines and column are rapidly washed with acetonitrile
Capping of the column:			
Wash A	20	0	System is flushed with anhydrous acetonitrile
Cap A and B	7	0	Equal volumes of cap A and cap B solutions are rapidly delivered to column
Cap A and B	6	15	Slower delivery of cap A and cap B to column (to maximize capping efficiency)
Wash A	6	15	Slow delivery of acetonitrile to column to purge remaining cap A and B solutions
Wash A	14	0	Lines and column are rapidly flushed with anhydrous acetonitrile
Sulfurization of phosphoramidite:			
Sox	15	0	Sulfurizing reagent is rapidly pulsed through column to sulfurize the newly formed phosphite triester linkages
Sox	25	400	Slower delivery of sulfurizing reagent through column (to maximize sulfur transfer efficiency)
Wash A	15	200	Slow delivery of acetonitrile to column to purge remaining sulfurizing solution from system
Wash A	15	0	System is rapidly flushed with acetonitrile
Capping of the column:			
Cap A and B	7	0	Equal volumes of cap A and B solutions are rapidly delivered to column
Wash A	30	0	Lines and column are flushed with anhydrous acetonitrile

[a]Standard DNA coupling cycles supplied by the manufacturer have been optimized for 2′-araF oligonucleotide assembly.
[b]Represents the number of pulses required for the corresponding step; each pulse has approximately 16 μL volume.

2. Weigh out the calculated amounts of phosphoramidites into the appropriate synthesizer vials and leave these to dry in a vacuum desiccator over phosphorus pentoxide overnight.

3. Flush the desiccator with argon gas before opening, and carefully remove the preweighed amidites.

4. Dissolve the phosphoramidites in anhydrous acetonitrile to a final concentration of 50 mg/mL.

   Use of syringe to transfer the acetonitrile through the rubber septum of each capped synthesizer vial to ensure the reagents are protected from humidity. Do not open the phosphoramidite vials until they are ready to be placed directly on the synthesizer.

5. Connect the solutions from the Liquid Reagents kit for the Expedite 8909 instrument, the phosphoramidite solutions, and the sulfurization reagent to the automated DNA synthesizer according to the manufacturer's directions.

   The Expedite 8909 synthesizer is equipped with an extra position for the sulfur transfer reagent. If a synthesizer without this option is to be used, the sulfurization reagent may be placed at the position of the oxidizer, provided that the line has previously been washed with acetonitrile to avoid cross-contamination of the reagents.

6. Purge the lines of the synthesizer with all the solutions and solvents.

7. Install the appropriate amidite column.

8. Modify the synthetic cycles for araF phosphoramidites. Examples of synthesis cycles for phosphodiester and phosphorothioate araF oligonucleotides are given in Table 4.15.1 and Table 4.15.2.

9. Enter the sequence to be synthesized.

10. Carry out the assembly on the instrument according to the manufacturer's instructions, choosing the "DMTr-off" (trityl-off) option.

11. Check the coupling efficiency periodically using the trityl monitor equipped with the instrument (also see *APPENDIX 3C*).

12. When the synthesis is complete, remove the column from the synthesizer and dry it under vacuum.

13. Deprotect and purify to obtain the desired oligonucleotide (see Basic Protocol 3).

BASIC PROTOCOL 3

DEPROTECTION AND PURIFICATION OF araF OLIGONUCLEOTIDES

This protocol describes the steps necessary for cleavage of araF sequences, assembled as described above (see Basic Protocol 2), from the solid support and removal of the protecting groups from the heterocyclic bases and phosphates. The procedure for the quantitation of isolated oligonucleotides is also described.

AraF oligonucleotides can be successfully analyzed and purified by anion-exchange HPLC or denaturing gel electrophoresis (PAGE; *UNIT 10.4* and *APPENDIX 3B*), as described below. Analytical amounts of material can easily be isolated by gel electrophoresis, while large amounts require HPLC purification. Desalting of the oligomers after gel or chromatographic isolation completes the purification procedure. There are two different methods that may be employed to accomplish this, either size-exclusion chromatography or reversed-phase SepPak C18 cartridge purification.

Materials

Fully protected oligonucleotides, attached to the solid support of a synthesis column (see Basic Protocol 2)
Ethanol, anhydrous
29% ammonium hydroxide
Denaturing acrylamide gel stock solution (see recipe)
Loading buffer (e.g., formamide/dye mix, *UNIT 10.4*)
Sephadex G-25 column (Amersham Pharmacia Biotech)
1 M $NaClO_4$
Anhydrous and 25% or 50% (v/v) acetonitrile

1.5 mL microcentrifuge tubes or screw-cap microcentrifuge tubes with O-ring seal
Platform shaker
55°C heating block or water bath (optional)
Vacuum evaporator (e.g., Savant Speedvac) with low and high vacuum sources
UV spectrophotometer and cuvette
0.75- and 1.5-mm-thick gel plates
TLC Merck silica plate (Kieselgel 60 F-254)
Hand-held 254-nm UV lamp
Camera with UV filter (optional)
0.45 µm hydrophilic fluid filter (Creative Medical)
0.22 µm membrane filter (Millipore; optional)
Anion-exchange high-performance liquid chromatograph (HPLC) with:
  Gradient maker
  0.5 mm × 7.5 cm Protein Pak DEAE 5PW column (Waters)
  Column heater
Sep-Pak C18 cartridges (Waters Chromatography)
10 mL syringe

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (*APPENDIX 3B* and *UNIT 10.4*)

Deprotect oligonucleotides

1. Remove the fully protected oligonucleotides attached to the solid support of the synthesis column, and place in a 1.5 mL microcentrifuge tube or a screw-cap microcentrifuge tube with O-ring seal (seal tightly) if deprotecting at increased speed and temperature (step 3).
2. Add 0.25 mL anhydrous ethanol and 0.75 mL of 29% ammonium hydroxide.
3. Place the tube on a platform shaker for 48 hr at room temperature. Alternatively, deprotect by incubating in a 55°C heating block or waterbath for 15 hr.
4. Once the deprotection is complete, cool the microcentrifuge tube ~1 hr in the freezer (i.e., –20°C) before opening.
5. Microcentrifuge the samples briefly so that the CPG beads are allowed to settle, then transfer the supernatant to another microcentrifuge tube. Wash the CPG with ethanol twice and place the supernatant from each wash in additional microcentrifuge tubes.
6. Dry the samples in a vacuum evaporator, applying low vacuum first to remove all traces of ammonia.

   Introducing high vacuum to the ammonia/ethanol solution may cause bumping of the liquid and loss of the sample.

7. When the ammonia has been evaporated and sample volumes are decreased enough, combine the supernatants from each microcentrifuge tube. Evaporate the final sample to dryness under high vacuum.

Quantitate oligonucleotide

8. Dissolve the oligonucleotide from the previous step in 1 mL deionized water. Remove a 10-µL aliquot and dilute this to 1 mL with deionized water in a UV cuvette.

9. Measure the absorption of the sample at 260 nm. Calculate the amount of crude oligonucleotide obtained in A_{260} units.

   Typically, a 1-µmol synthesis may give yields of 100 to 180 A_{260} units of crude material, depending on the sequence length and composition.

Analyze crude oligonucleotide by denaturing PAGE

10. Prepare a 24% denaturing polyacrylamide gel on a 0.75-mm-thick analytical gel plate for gel electrophoresis (*APPENDIX 3B* and *UNIT 10.4*).

11. Prepare samples of oligonucleotides to be analyzed (e.g., 0.5 to 1 A_{260} units per analysis). Dissolve each of these in 10 µL loading buffer.

12. Load the samples into wells and run these alongside loading buffer alone, which is placed in the first and last lanes of the gel. Connect the gel to the power supply and start running at 200 V until the dye has fully diffused into the gel. Increase the voltage to 500 V and maintain at this setting until the faster running dye marker has traveled ¾ down the length of the gel.

13. Turn off the current and dismantle the gel apparatus. Put the gel in plastic wrap. Place a fluorescing TLC Merck silica plate under the gel and examine by illumination with a hand-held 254-nm UV lamp. If necessary, photograph the gel using a camera with a UV filter.

    Oligonucleotides absorb UV light and appear as dark bands in the gel against a fluorescent background. Typically, crude FANA oligonucleotides, both diester and thio, consist of one intense band. The presence of multiple bands below the desired product usually indicates poor coupling efficiency.

Purify oligonucleotides by denaturing PAGE and desalt using Sephadex G-25

14. Prepare a 24% to 16% denaturing polyacrylamide gel on a 1.5-mm-thick gel plate for preparative analysis (*APPENDIX 3B* and *UNIT 10.4*).

    Compared to analytical separations, a lower percentage of acrylamide may be used in preparative analyses (from 16% to 24%), depending on the quality of the synthesis. For example, a crude oligonucleotide which migrates as one main band in analytical compositions can be sufficiently purified using 16% acrylamide in the preparative run. When multiple bands corresponding to failed sequences are present in the gel, a higher percentage of acrylamide solution should be used.

15. Prepare and dry the oligonucleotide sample to be purified (~20 to 30 A_{260} units). Dissolve the sample in 100 µL loading buffer.

    Sample loading to the gel may vary, depending on the quality of the synthesis.

16. Load the sample on the gel and repeat steps 12 and 13 above.

17. Using a razor blade, cut out the segment of gel containing the band of interest.

18. Transfer the excised band to a 15-mL conical plastic tube with screw cap and crush with a spatula. Add 10 mL water and leave the tube on a platform shaker overnight to extract the oligonucleotide from the gel.

19. Filter the supernatant through a 0.45-µm hydrophilic fluid filter and concentrate on a vacuum evaporator.

20. Desalt the sample using a Sephadex G-25 column according to the manufacturer's directions.

21. Quantitate the amount of oligonucleotide isolated via UV spectroscopy.

22. Lyophilize the oligonucleotide.

    The purified, dry oligonucleotide can be stored for long periods (e.g., >1 yr) at −20°C. If the oligonucleotide is to be used in combination with microbially sensitive materials (e.g., cell culture), filtration through a 0.22-µm filter is recommended. Collect the filtrate in previously autoclaved tubes and lyophilize.

Analyze and purify oligonucleotide by anion-exchange HPLC and desalt on SepPak C18 cartridge

23. Prepare the anion-exchange HPLC by setting the detector wavelength to 260 nm, heating the column to 50°C, and equilibrating with buffer A (deionized water) using a flow rate of 1 mL/min or as specified by the manufacturer.

    See UNIT 10.5 and legends to Figures 4.15.2 to 4.15.4 for more details.

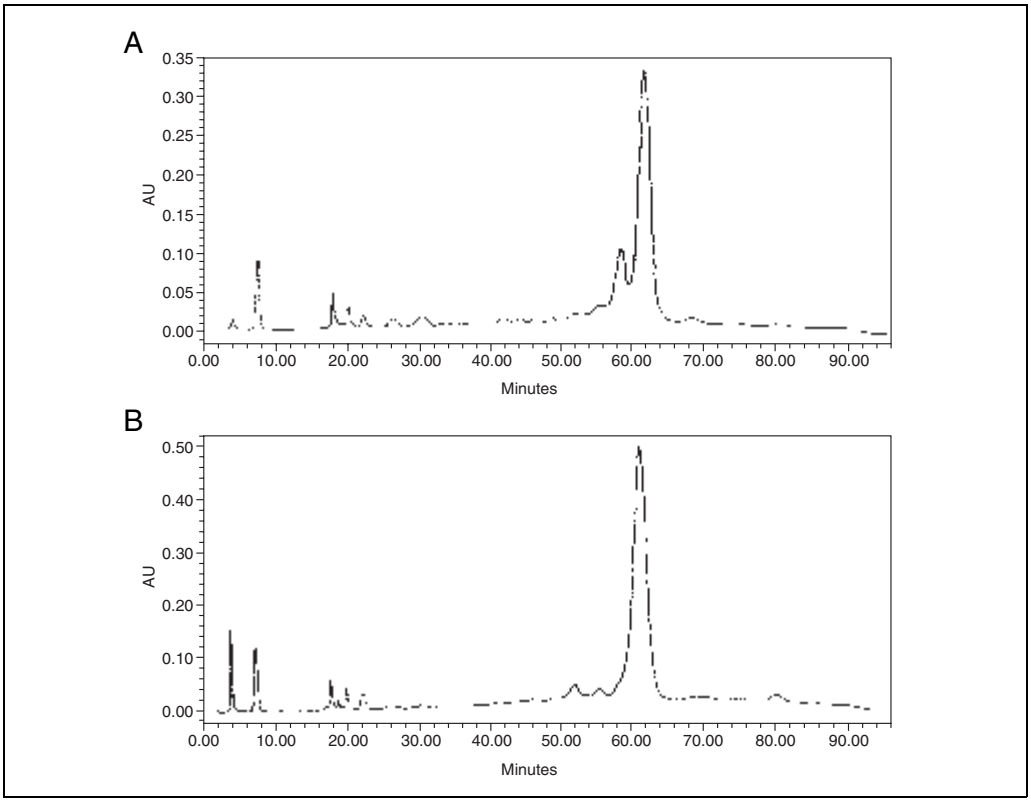

Figure 4.15.2 Ion-exchange HPLC analysis of crude S-oligonucleotide ATA TCC TTg TCg TAT CCC (cap letters represent araF nucleotides, DNA residues in lowercase). (**A**) Sulfurization with Beaucage reagent. The small peak at ~58 min represents S-oligonucleotides with one P-O insertion. The main peak at ~62 min represents full S-FANA. (**B**) Sulfurization with ADTT. System: Waters 600E Multisolvent Delivery System with Waters 486 Tunable Absorbance detector and oven, driven by Millennium (V 3.20) software; column: Waters 0.5-mm × 7.5-cm Protein Pak DEAE 5PW, 50°C; solvent A: H_2O; solvent B: 1 M $NaClO_4$; flow rate: 1 mL/min; detection: 260 nm; gradient conditions: 0% B for 9 min, 0% to 15% B in 3 min, 15% to 50% B in 60 min, 50% to 80% B in 2 min, hold 80% B 10 min, 80% to 0% B in 2 min, hold 0% B 10 min.

24. Load ~1 A_{260} unit crude oligonucleotide sample (steps 8 and 9) to the column. Wash 5 min with buffer A, then apply a linear gradient of 0% to 25% buffer B (1M $NaClO_4$) in 60 min (i.e., 0.42%/min) to elute diester araF oligonucleotides, or of 0% to 50% buffer B in 60 min (i.e., 0.83%/min) for S-FANA oligonucleotides. Wash the column and re-equilibrate.

The desired fraction should elute close to the end of the applied gradient.

25. Optimize the conditions for the next run. Examples of suitable chromatographic gradients that may be used are given in the legends to Figures 4.15.2 to 4.15.4.

26. Continue purification of the next portions of oligonucleotide using the previously optimized conditions. Increase the amount of oligonucleotide loaded to the column and set the detector at 280 to 290 nm depending on the amount of the sample to be loaded.

Use higher wavelengths for larger samples to avoid saturating the UV detector. Sample loading to the column depends on the quality of the reaction mixture and type of oligonucleotide. Typical loading amounts for phosphodiester FANA oligonucleotides range from 10 to 30 A_{260} units of crude reaction mixture using the conditions described above. Loading for S-FANA oligonucleotides can be increased considerably as a result of their stronger adsorption on the ion-exchange column. The authors have been able to purify ~100 A_{260} units of crude mixture in one loading.

27. Quantitate the total amount of oligonucleotide obtained.

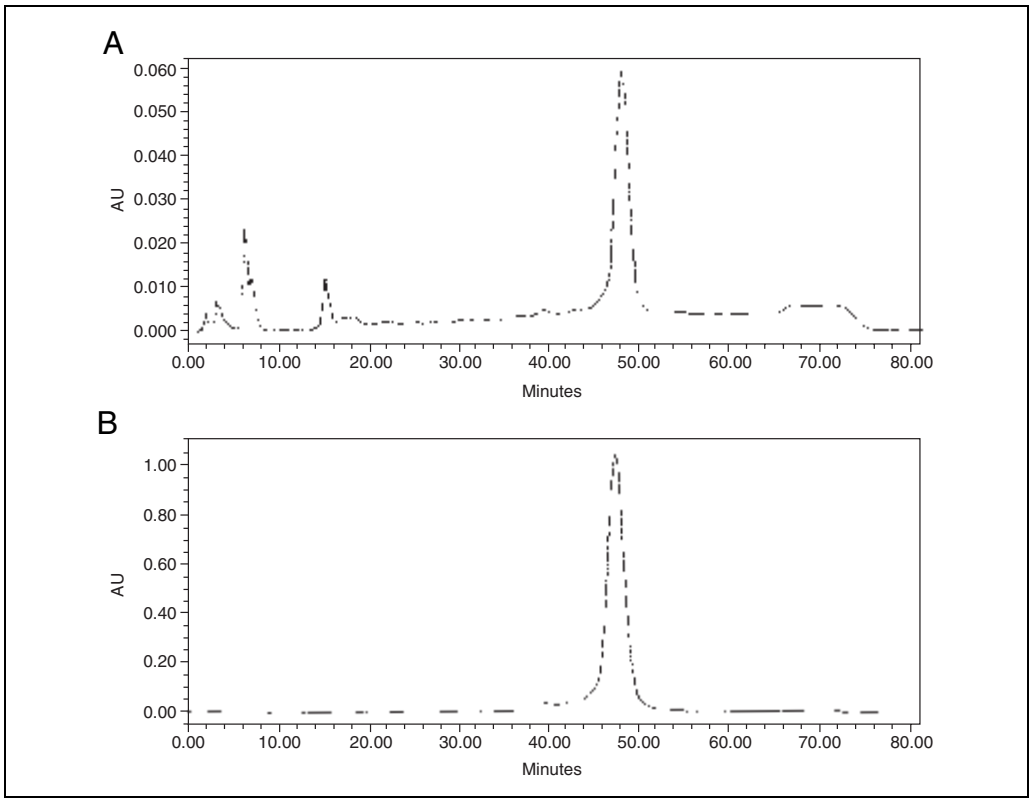

Figure 4.15.3 Ion-exchange HPLC analysis of (**A**) crude and (**B**) purified S-oligonucleotide CTC TAg cgt ctT AAA (cap letters represent araF nucleotides, lowercase letters represent DNA residues). System: Waters Binary HPLC Pump with Waters 2487 Dual λ Absorbance detector, equipped with in-line degasser and oven, driven by Breeze (V 3.20) software; column: Waters 0.5-mm × 7.5-cm Protein Pak DEAE 5PW, 50°C; solvent A: H_2O; solvent B: 1 M $NaClO_4$; flow rate: 1 mL/min; detection: 260 nm; gradient conditions: 0% B for 9 min, 0% to 15% B in 3 min, 15% to 50% B in 45 min, 50% to 80% B in 2 min, hold 80% B 10 min, 80% to 0% B in 2 min, hold 0% B 10 min. Peaks at <20 min correspond to failure sequences and loss of protecting groups (e.g., benzoyl from A or C).

28. For the desalting step, prepare SepPak C18 cartridges first by washing them with 10 mL (each) water, then anhydrous acetonitrile, then water, using a 10-mL syringe.

    Make sure that the cartridges are well equilibrated with water; traces of acetonitrile will prevent adsorption of the oligonucleotide and may lead to substantial loss of material.

29. Load ~20 to 30 A_{260} units of oligonucleotide per cartridge, wash the cartridge with 10 mL water, and then elute the pure desalted oligonucleotide with 5 mL of 25% or 50% acetonitrile in water for phosphodiester and phosphorothioate-oligonucleotides, respectively. Quantitate the amount of oligonucleotide obtained.

    Always collect water fractions from all desalting steps. If the cartridge was not equilibrated properly or was overloaded, the oligonucleotide may elute with the water fraction. If this occurs, load the water fraction with oligonucleotide to a new cartridge and repeat the desalting procedure.

30. Evaporate the oligonucleotide on a vacuum evaporator using low vacuum first to remove acetonitrile, followed by complete drying of the sample under high vacuum.

31. If necessary, filter the oligonucleotide through a 0.22 µm membrane filter (see comments in step 22).

    Desalting after HPLC purification can be done on Sephadex G-25 as well.

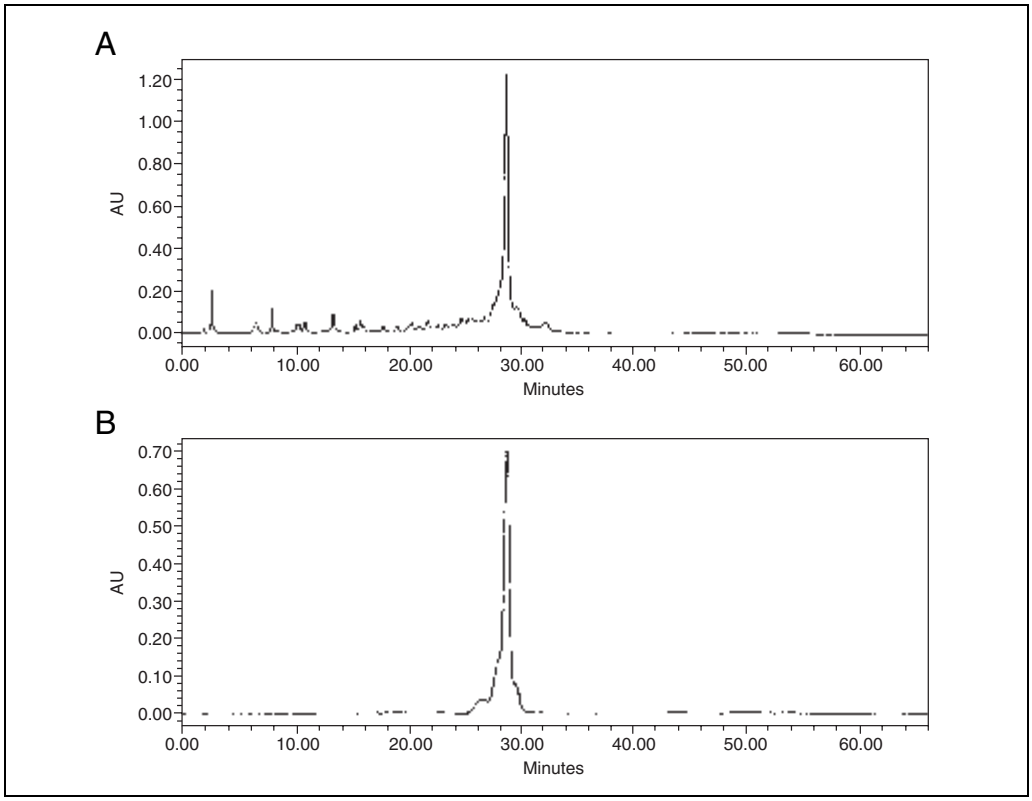

Figure 4.15.4 Ion-exchange HPLC analysis of (**A**) crude and (**B**) purified phosphodiester oligonucleotide ATg TCC TTg TCg gTg Agg TTA GG (cap letters represent araF nucleotides, lowercase for DNA residues). System: Waters Binary HPLC Pump with Waters 2487 Dual λ Absorbance detector, equipped with in-line degasser and oven, driven by Breeze (V 3.20) software; column: Waters 0.5-mm × 7.5-cm Protein Pak DEAE 5PW, 50°C; solvent A: H_2O; solvent B: 1 M $NaClO_4$; flow rate: 1 mL/min; detection: 260 nm; gradient conditions: hold 0% B 2 min, from 0% to 30% B in 40 min, from 30% to 45% B in 2 min, hold 45% B 10 min, 45% to 0% B in 2 min, hold 0% B 10 min.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acetonitrile, anhydrous

Predry acetonitrile by refluxing and distilling over phosphorus pentoxide overnight. Reflux and distill over calcium hydroxide. Store sealed or on the synthesizer up to 1 month at room temperature.

Amidite column

Prepare a succinyl-LCAA-CPG support derivatized with 5′-(4-monomethoxytrityl) and the base-protected araF nucleoside of choice (**S.1a-d**; *UNIT 3.2*). Average nucleoside loadings are 20 to 40 μmol/g, according to trityl analysis in *APPENDIX 3C.*

Cap A

10 mL acetic anhydride
10 mL 2,6-lutidine or 2,4,6-collidine
80 mL THF (see recipe)
Store sealed or on synthesizer up to 1 month at room temperature

Cap B

16 mL *N*-methylimidazole
84 mL THF (see recipe)
Store sealed or on synthesizer up to 1 month at room temperature

Denaturing acrylamide gel stock solution

For 24% analytical gel:
120 g acrylamide
6 g *N*, *N*′-methylene-bisacrylamide
210 g urea
50 mL 10× TBE electrophoresis buffer (*APPENDIX 2A*)
Water to 500 mL
Store protected from light for up to 1 year at 4°C.

Immediately before pouring, degas 50 mL stock solution for 10 min and add 20 μL TEMED and 200 μL freshly prepared 10% (w/v) ammonium persulfate. Allow gel to polymerize overnight at room temperature (also see *APPENDIX 3B* and *UNIT 10.4* for pouring acrylamide gels).

For 16% to 24% preparative gel:

Prepare stock solution and gel as above, but use 80 g acrylaminde and 4 g *N*,*N*′-methylenebisacrylamide for 16% gel, or scale as appropriate for up to 24%. Immediately before pouring, degas 65 mL stock solution for 10 min and add 35 μL TEMED and 350 μL freshly prepared 10% (w/v) ammonium persulfate.

Oxidizer solution

1.23 g iodine
25 mL THF (see recipe)
20 mL pyridine
2 mL H_2O
Store sealed up to 6 months at room temperature or on synthesizer up to 2 wks at room temperature.

Sulfurization reagent

Any of the reagents listed below can be used to sulfurize the phosphite triester linkage.

Beaucage reagent:

2 g 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (0.2 M)

50 mL anhydrous acetonitrile (see recipe)

Store on the synthesizer up to 2 or 3 days at room temperature. Discard if precipitate is observed.

EDITH reagent:

1 g 3-ethoxy-1,2,4-dithiazoline-5-one (0.125 M)

100 mL anhydrous acetonitrile (see recipe)

Store up to 1 month at room temperature

ADTT reagent:

1.5 g 3-amino-1,2,4-dithiazoline-5-thione (0.1 M)

50 mL anhydrous acetonitrile (see recipe)

50 mL pyridine

Store up to 1 month at room temperature

Tetrahydrofuran (THF), anhydrous

Dry tetrahydrofuran by distillation over sodium and benzophenone. Storage of anhydrous THF is not recommended as peroxides may be formed. Always use freshly distilled.

COMMENTARY

Background Information

Antisense oligonucleotides (AONs) represent an extensive class of biologically relevant, nonnatural polymeric nucleic acids that have potential utility as novel pharmaceuticals or in molecular diagnostics applications as probes of gene function. These analogs are specifically designed to induce selective gene interference via tight and specific associations with their intended genetic targets. For example, such a "bottleneck" approach can target the mRNA directly to inhibit the expression of a dysfunctional gene, and thereby transcend traditional approaches that control a disease phenotype by targeting the malformed protein (for review, see Myers and Dean, 2000, and references therein). Furthermore, with the complete sequence of the human genome now at hand, the future design of antisense-based therapeutics may be feasible without prior knowledge of the protein structure.

Indeed, adequate optimization of the biological properties of chemically modified AONs is paramount to the success of this and related technologies. The most efficacious AON members are those that ideally possess remarkable stabilities against both general serum and cellular exo- and endonucleolytic activities, and adequate lipophilicity to ensure efficient cell permeation. They should also exhibit high discrimination toward fully complementary host cell sequences and allow minimal residual expression of the target gene (see Lebedeva and Stein, 2001, and references therein).

Additionally, suppression of gene activity in eukaryotic organisms may proceed catalytically with the assistance of intracellular ribonuclease H (RNase H), a ubiquitous enzyme implicated in DNA replication and repair processes (Walder and Walder, 1988; Crouch and Toulmé, 1998). In fact, the extent to which protein synthesis is inhibited via antisense action in vivo seems to correlate with the propensity of the preformed AON:RNA substrate to elicit RNase H destruction of the mRNA component. Thus, a single enzyme-active AON can be recycled by this pathway to silence multiple copies of an RNA transcript, and display superior inhibition to those that do not activate the enzyme (Uhlmann and Peyman, 1990). Importantly, these events hinge upon presentation of the correct hybrid shape by the substrate duplex to the enzyme for subsequent hydrolysis of the targeted species (Nakamura et al., 1991; Oda et al., 1993). However, most AONs lack the appropriate structural attributes for enzyme elicitation and typically display less than optimal cellular potencies (see for example, Lima and Crooke, 1997).

Table 4.15.3 Thermal and Biological Properties of Oligonucleotide Analogs[a]

Oligonucleotide analogue	ΔT_m/mod. (°C)	RNase H activation	Reference
thioate-LNA	+10.0	No	Wengel et al., 1999
LNA	+5.6	No	Wengel et al., 1999
α-L-LNA	+4.6	Yes	Sørensen et al., 2002
2′F-RNA	+3.0	No	Manoharan, 1999
N-methanocarba-NA	+2.6	n/a[b]	Altmann et al., 1994a
MMI-2′OMe-RNA	+2.0	No	Sangvhi, 1998
2′-MOE-RNA	+2.0	No	Manoharan, 1999
2′OMe-RNA	+1.7	No	Manoharan, 1999
RNA	+0.7	No	Wilds and Damha, 2000
CeNA	+2	Yes	Wang et al., 2000
2′F-ANA	+1.2	Yes	Wilds and Damha, 2000
thioate-2′F-ANA	+0.5	Yes	Lok et al., 2002
DNA	+0.5	Yes	Wilds and Damha, 2000
thioate-DNA	0	Yes	Wilds and Damha, 2000
ANA	–0.5	Yes	Noronha et al., 2000
S-methanocarba-NA	–0.6	n/a[b]	Altmann et al., 1994b
2′β-Me-ANA	–2.8	No	Schmit et al., 1994
[3.3.0]bc-ANA	–3.5[c]	No	Christensen et al., 1998

[a]Abbreviations: α-L-LNA, α-L-*ribo*-configured locked nucleic acid; 2′β-Me-ANA, 2′-deoxy-2′-O-methylarabinonucleic acid; ANA, arabinonucleic acid; [3.3.0]bc-ANA, 2′,3′-[3.3.0]-bicycloarabinonucleic acid; CeNA, cyclohexene nucleic acid; 2′F-ANA, 2′-deoxy-2′fluoroarabinonucleic acid; 2′F-RNA, 2′-deoxy-2′-fluororibonucleic acid; LNA, locked nucleic acid; *N*-methanocarba-NA, *Northern*-locked 4′,6′-methanocarbocyclic nucleic acid; *S*-methanocarba-NA, *Southern*-locked 1′,6′-methanocarbocyclic nucleic acid; MMI-2′OMe-RNA, methylene(methylimino)-2′-OMe-RNA; 2′-MOE-RNA, 2-O-(2-methoxyethyl) RNA; 2′OMe-RNA, 2′-methoxyribonucleic acid; thioate-DNA, phosphorothioate DNA; thioate-2′F-ANA, phosphorothioate 2′-deoxy-2′fluoroarabinonucleic acid; DNA, deoxyribonucleic acid; thioate-LNA, phosphorothioate locked nucleic acid.
[b]Data not available.
[c]Reported for the mixed base deoxy 9mer with 3 dispersed bc-ANA inserts; note that contiguous bc-ANA insertion enhances duplex stability up to +1.7°C / mod. relative to thioate-DNA.

An important parameter that governs overall duplex geometry is the conformations adopted by the individual sugars in the AON. To this end, modifications at the 2′-position have proven most valuable as tools for influencing ring conformational equilibria. Additionally, much of the literature devoted to antisense modifications has shown that crude predictions of sugar gauche and anomeric effects can be made with some accuracy as to the effect a particular 2′-modification exerts on the pseudo-sugar equilibrium (e.g., Plavec et al., 1994; Thibaudeau et al., 1994; Damha et al., 1995; Thibaudeau and Chattopadhyaya, 1997; Noronha and Damha, 1998; Wilds and Damha, 1999).

The authors' evaluation of these effects indicated to them that a switch in chirality of the α-2′-OH group in ribose residues to the β-configuration in the arabinose epimers should drive the N-to-S equilibrium to the southern hemisphere and enforce a noncanonical A:B topology in hybrids with RNA, in common with the native RNA:DNA hybrids. It is noteworthy that most 2′-modifications do not activate RNase H, as their double-stranded helices with RNA are tailored to adopt a pure A form—i.e., RNA:RNA-like (for reviews, see Manoharan, 1999, and references therein). In fact, arabinonucleic acids (ANAs) represent the first examples of sugar-modified antisense agents capable of eliciting RNase H–mediated hydrolysis of target RNA.

Additionally, ANAs exhibit adequate hydrolytic stability as a result of the trans relationship of the 2′- and 3′-OH groups, which effectively retards internal nucleophilic participation of the 2′-OH group in chain isomerization and/or phosphodiester cleavage. Despite these appealing properties, oligoarabinonucleotides of

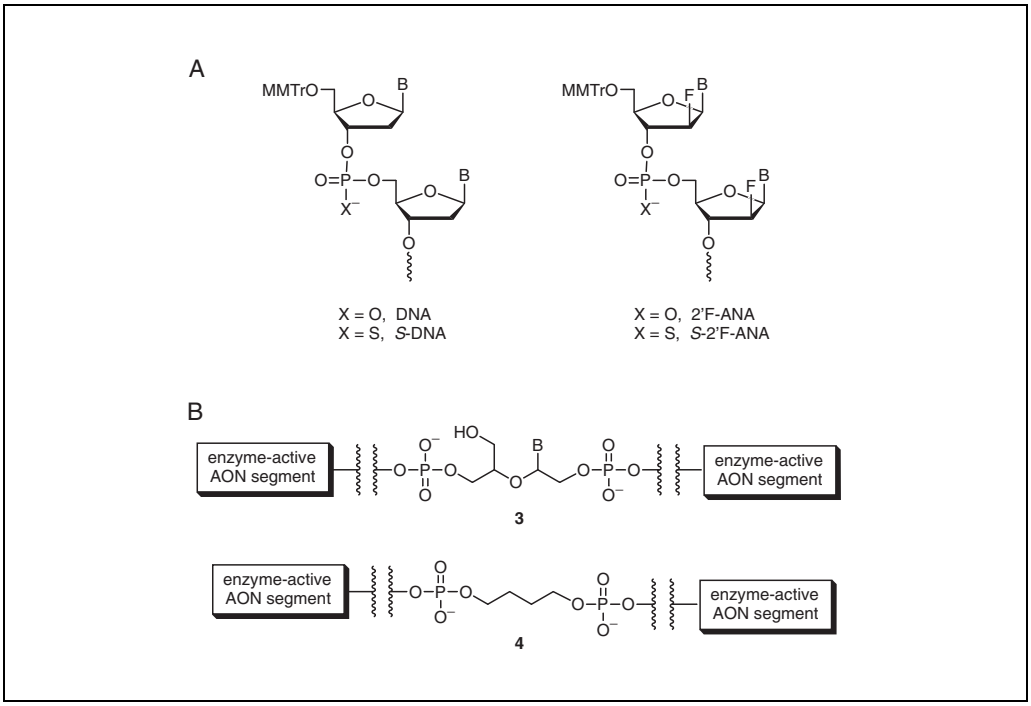

Figure 4.15.5 (**A**) DNA and 2′F-ANA primary structures and backbone chemistries. (**B**) Illustration of linker-modified DNA or 2′F-ANA antisense constructs. Panel B reprinted from Mangos and Damha (2002) with permission from Bentham Science Publishers.

mixed base composition display weaker binding affinities relative to the native DNA:RNA heteroduplexes (Giannaris and Damha, 1994; Damha et al., 1998; Noronha et al., 2000). The observed thermal destabilization may arise from cis-placement of the sterically demanding β-C2′-OH group with respect to the base in the major groove of the helix, which purportedly weakens local π-stacking and/or normal Watson-Crick pairing by distorting the *N*-glycosyl orientation (Noronha et al., 2000). Alternatively, molecular dynamics simulations have suggested the arabinose sugars to be held in C2′-endo geometries by probable intrasugar hydrogen bonding between the 2′-α-substituent and the C5′-oxygen (Venkateswarlu and Ferguson, 1999). The strand topology is consequently forced to adopt a B-like conformation, which is disfavored for binding with RNA. This, however, contradicts recent NMR analyses (Trempe et al., 2001), which show a clear bias of the sugar conformation toward the eastern (O4′-endo) hemisphere and a lack of C2′-OH/O5′ interactions in the arabinose sugars (Damha et al., 2001).

Interestingly, the CD spectral profiles of DNA:RNA and ANA:RNA complexes reveal similar duplex helicities; however, the ability of the latter to direct RNase H–mediated phosphodiester hydrolysis of the target occurs with comparably lower efficiencies. This lower processivity of the enzyme toward ANA:RNA hybrids may arise from a weaker tenacity of ANA for RNA targets and a correspondingly lower effective concentration of substrate duplex. Accordingly, the extent of cleavage increases significantly when substrate and enzyme are incubated at ambient rather than physiological temperatures (Noronha et al., 2000).

Apart from conferring greater glycosyl stability towards potential depurination, a β-F atom in place of the ara-2′-OH group (i.e., 2′F-ANA) markedly increases the duplex melting temperature (Damha et al., 1998), imparting higher stability than hybrids of RNA and ANA, thioate-DNA or even DNA (Table 4.15.3). The origin of these effects likely stems from greater strand preorganization of the fluorinated strands relative to their single-stranded DNA counterparts. In fact, simple substitution with the more electronegative F atom almost exclusively forces the arabino-sugar equilibrium to adopt an O4′-endo (or eastern) conformation (Berger et al., 1998; Trempe et al., 2001). Like the arabinose compounds, this predominance over a C2′-endo geometry is presumably forced to occur in order to minimize steric conflicts between the F atom and the proximal heterocycle (Berger et al., 1998). Rather, the 2′-substituent is conveniently accommodated in the major groove

by assuming a more eastern geometry, which displaces it sufficiently far from the enzyme's locus of action while retaining other geometrical elements necessary for activity. In fact, 2′F-ANA, exhibits superior RNase H competency relative to ANA, probably by virtue of the sterically innocuous properties of fluorine and the higher thermal stability of FANA/RNA hybrids (Damha et al., 2001).

Although much conformational work has been directed to the phosphodiester analogs (vide supra), many of these observations can be extended to 2′F-ANA oligomers with phosphorothioate (S) scaffolds. The dominant influence of fluorine on sugar-pucker profiles and subsequent enzyme hydrolytic susceptibility is retained in S-FANA (Lok et al., 2002), in which additional nuclease resistance is provided to the 2′-modified oligomers by the thioate backbone (reviewed by Cook, 1998).

However, uniformly modified S-FANA containing hybrids are suboptimally recognized by RNase H as compared to their phosphodiester derivatives, but can greatly be improved with deoxy incorporation (Lok et al., 2002). Likewise, other oligonucleotide modifications that combine intervening enzyme-active AON segments with conformationally restricted "flanking" residues usually support cleavage of their intended targets. The peripheral segments also serve to provide added nuclease resistance to the core segment, which is usually a stretch of nuclease-labile deoxynucleotides. Although the entropic consequences imposed by the flanking segments on the antisense strand endow it with favorable duplexation properties, RNase-H-induction is invariably compromised (Shen et al., 1998). Accordingly, non-RNase H competent flanking backbone segments (e.g., 2′OMe-RNA) show strong dependencies on gap size, with a minimal requirement of six to eight contiguous deoxynucleotides within the core to elicit observable activities. These requirements are significantly relaxed for S-FANA in which insertion of even a single deoxynucleotide as the "gap" restores high in vitro activities. In fact, fluoroarabino-deoxy chimeras comprising four or more neighboring deoxynucleotides exhibit remarkably greater potencies than uniform S-DNA sequences and show greater inhibition of luciferase expression in HeLa cells without affecting cell viability (Lok et al., 2002). Furthermore, cleavage occurs throughout the entire RNA chain, rather than specifically within the gap as for the methylated ribosyl congeners, which adopt a characteristic C3′-endo pucker and likely induce local helical deformations spanning the methoxy regions of the AON:RNA chimera hybrids.

These results further highlight the pivotal importance of the O4′-endo antipodal conformation as a major determinant for RNase H recognition. Consequently, the most promising antisense agents are hypothesized to be those in which the arabinose configuration at C2′ is retained (Venkateswarlu and Ferguson, 1999). However, other carbohydrate modified ANA derivatives with this same pseudosugar disposition have displayed poor RNase H–associated hydrolysis of target RNA. Significantly, various bicyclic ANAs possess an O4′-endo bias and could hypothetically induce RNase H at high concentrations, but generally show abolished activities under conditions representative of the intracellular environment (Minasov et al., 2000). Interestingly, the inherent flexibility of the sugar conformations strongly correlate with the relative processivity of the enzyme toward hybrids of ANA-derived AON and RNA according to the following: DNA (flexible) > 2′F-ANA/ANA >> bc-ANA (rigid). These trends, together with the activity enhancements observed upon introducing relatively flexible deoxy residues in the more rigid FANA strand (vide supra), prompted the authors to investigate the potential synergistic attributes of intermingling sites of flexibility within the 2′F-ANA polymers.

Indeed, the incorporation of acyclic linkers with high degrees of flexibility in the AON strand, either a 2′,5′-linked secouridine residue **S.3** or a butanediol linker **S.4** (Figure 4.15.5), appear to better accommodate the stringent conformational requirements of the enzyme without disrupting other fundamental recognition elements within the enclosing AON segments (Mangos et al., unpub. observ.). Sizable increases in enzyme-mediated scission of target RNA occur upon substituting an acyclic linker in the middle of a known RNase H–competent analog (i.e., DNA or 2′F-ANA). Moreover, the susceptibility of the hybrid to RNA hydrolysis remains fully operative in other sequence contexts with up to eight-fold enhancements over strands lacking the acyclic insertion. For example, in sequences wherein the acyclic inserts are moved along the 2′F-ANA scaffold, a significant change in activity is observed that depends specifically on the insertion site. The greatest activity occurs in constructs with a centrally placed acyclic linker, although differences between 5′- versus 3′-end insertion are also observed, with a greater amount of enzyme proc-

essing occurring when the linker is placed near the 3′-end of the AON. The authors therefore speculate that RNase H induction can remarkably be improved by introducing subtle changes in local 2′F-ANA strand dynamics that enable better adherence and/or processing of the hybrid substrate by the enzyme. These and future studies with 2′F-ANA and their constructs should provide considerable insights as to the structural factors that comprise the "optimal" AON/RNA substrate.

For these reasons, the protocols of this unit provide a method of quickly and conveniently preparing 2′-fluoroarabino oligonucleotides with any one or all of the structural modifications described herein. Although linker technologies are not discussed, these are quite simple to implement and exemplify the diversity with which the protocols can be applied to new AON designs that further exploit the interesting properties of 2′F-ANA.

Critical Parameters

The synthesis of araF-phosphoramidites, as for other phosphoramidites, is very moisture and acid sensitive. Therefore, all reagents and apparatus should be anhydrous. If the product has to be isolated by flash column chromatography (*APPENDIX 3E*), it is absolutely necessary to include triethylamine in all solvent systems used to equilibrate the column and for subsequent sample elution. Heating the product above 40°C is not recommended during evaporation. If the phosphoramidite has been stored for a long period of time prior to oligomer synthesis, its purity should be verified by TLC (*APPENDIX 3D*) or ^{31}P NMR. If necessary, the phosphoramidite can be repurified by flash chromatography.

Phosphoramidite oligonucleotide chemistry is extremely water sensitive. All coupling reagents should be absolutely dry, and fresh reagents should be prepared as these are critical to the success of the synthesis. It is not recommended to keep the reagents on the synthesizer for longer than 1 week. Furthermore, the synthesizer should be in good working condition. All lines should be purged with each reagent and solvent prior to starting a synthesis and cleaned with acetonitrile afterward to prevent crystallization and blocking of the lines.

If synthesis is performed on the Expedite instrument, a relatively low concentration of phosphoramidites can be used without loss of coupling efficiency. For example, commercial RNA protocols require 50 mg/mL phosphoramidite concentration, which is almost 3-fold lower than for RNA synthesis on older instruments (e.g., 1.5 M amidite solutions are required for use with the ABI 381A DNA synthesizer). Phosphoramidite and activator solutions are continuously delivered to the column during the coupling step. Such permanent delivery is performed by slow pulses of reagents to the column, which permanently renew them and thereby increase the efficiency of the synthesis. If a synthesizer with stationary delivery of reagents is to be used, the amidite concentration should be increased to that used for RNA synthesis (or slightly lower). The authors were able to successfully synthesize diester araF oligonucleotides on the ABI 381A DNA instrument using 0.1 to 0.15 M concentrations of phosphoramidites.

Many different sulfur-transfer reagents for S-DNA have been documented in the literature over the last several years. However, none of these reagents are able to give 100% P-S oxidation. As a consequence, a small percentage of phosphodiester bonds are inevitably present in S-oligonucleotides. The diester fragments are much less stable to hydrolysis, making them more susceptible to nucleolytic cleavage in cell culture, which limits certain biological applications of these antisense oligonucleotides. Fortunately, pure S-oligonucleotides can easily be analyzed and purified from P-O containing S-oligonucleotides by anion-exchange HPLC (Bergot and Egan, 1992). This method enables the isolation of S-oligonucleotides that contain even a single P-O linkage within the strand. The percentage of P-O bonds in the product is dependent on the source of sulfurizing reagent used, as different sources may lead to different percentages in the fully synthesized oligomers. Although undesired oxidation is not very problematic in S-DNA synthesis, 2′F-ANA oligonucleotides are intrinsically more difficult to sulfurize as a consequence of the electron-withdrawing effects of the fluorine substituent in the sugars. This limitation may be overcome by increasing the concentration of the sulfur-transfer reagent, accompanied by extended reaction times in order to obtain S-oligonucleotides of better quality. The authors of the current protocol applied several sulfurization reagents to S-araF synthesis, namely the Beaucage reagent, EDITH, and ADTT, and have found that the former normally gives a higher percentage of P-O bonds relative to the latter two reagents. EDITH and ADTT work very similarly and give oligomers of low P-O compositions. Figure 4.15.2 shows a chromatographic profile for a crude S-DNA/S-araF chimera synthesized

using either the Beaucage reagent or ADTT. As is shown, the S-FANA oligonucleotide synthesized with ADTT displays more uniform P-S incorporation (i.e., P-O insertions are minimal in this oligomer as detected by chromatography). However, EDITH and ADTT reagents are very expensive and not widely available commercially, which makes the Beaucage reagent a suitable alternative. It should also be emphasized that the limitations observed with the Beaucage reagent are reserved only for 2′F-ANA polymers; experimentally, sulfurization with this reagent proceeds much more efficiently with other types of modified oligonucleotides (e.g., S-DNA), thereby making it the reagent of choice for those applications.

The amount of contaminating P-O linkages within S-FANA oligonucleotides does not typically exceed 1% per phosphate, even when using the Beaucage reagent, but may sometimes be significantly larger. Unfortunately, P-O insertions cannot be detected by PAGE. Oligonucleotides with multiple phosphodiester bonds will migrate as a single narrow band through the gel, but present multiple peaks upon anion-exchange chromatographic analysis. Consequently, chromatographic purification of S-FANA oligonucleotides is strongly recommended.

In general, both HPLC and PAGE purification can be applied to araF oligonucleotides. Sequences that consist entirely of phosphodiester backbones may be purified either by HPLC or PAGE, depending upon the scale used in the synthesis. If 1 to 20 A_{260} units are required, PAGE purification is sufficient; for preparative amounts ranging from 100 to 200 A_{260} units of purified oligomer, HPLC is recommended. For S-FANA oligonucleotides, ion-exchange purification is most convenient, regardless of the amount to be purified.

Troubleshooting

Low coupling efficiency. Several conditions can cause poor monomer coupling yields. (1) The reagents and acetonitrile may contain water. This is minimized by drying the phosphoramidites and tetrazole in a vacuum desiccator over phosphorus pentoxide for one or two days prior to use. (2) Lines in the synthesizer may be partially blocked. Check delivery of each reagent to the lines by monitoring flow rates. Perform routine DNA synthesis first and monitor coupling efficiencies by trityl analysis.

HPLC analysis of S-araF oligonucleotide gives multiple product peaks. This problem is usually caused by incomplete sulfurization, especially if the Beaucage reagent has been used. If so, the product may still migrate as one band under PAGE conditions. This can be circumvented by increasing the concentration of the Beaucage reagent or using another sulfurizing reagent.

Beaucage reagent precipitates. The Beaucage reagent is known to be extremely sensitive to the quality of solvent and is decomposed rather easily. If this process is rapid, a switch to an acetonitrile source of better quality is recommended. Furthermore, the glass bottle for Beaucage reagent should be silanized by the investigator (*APPENDIX 2A*; Iyer et al., 1990) or obtained in silanized form from various commercial sources. Alternatively, acetonitrile solutions of Beaucage reagent can be placed in a 15-mL plastic conical tube, which is then deposited within an appropriate glass bottle and attached to the gene-machine to prevent direct contact of the reagent with the glass.

Anticipated Results

Using araF phosphoramidites as building blocks in conjunction with the methods presented here, it should be possible to routinely obtain araF oligonucleotides with yields ranging from 120 to 180 A_{260} units of crude material for 1-μmol scale syntheses, and isolated yields of ~30 to 100 A_{260} units. The sulfurization step with EDITH or ADTT reagents helps to minimize the extent of P-O insertions in S-oligonucleotides. These considerations, together with the optimized ion-exchange HPLC chromatographic procedures, should enable the facile isolation of araF oligonucleotides of high purity.

Time Considerations

Preparation of araF phosphoramidites from the appropriately protected nucleosides usually takes ~1 day per monomer. Depending on the type of chemistry (diester- versus thio-araF-oligonucleotides, or DNA/araF oligonucleotide chimeras), the time required for assembly of a 20-mer oligonucleotide (typical for antisense applications) on the Expedite instrument is from 6 to 12 hr. An additional 2 days are required to perform deprotection, followed by 3 to 5 days for isolation, desalting, and final analysis as described (see Basic Protocol 3), if purification is required.

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